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DIAGNOSIS OF NATURAL AND INDUCED DISEASES OF MILITARY IMPORTANCE

FINAL, PHASE I REPORT

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13. ABSTRACT *(Maximum 200 words)*

The purpose of this research project was to demonstrate the feasibility of the Optical Bioassay (OBA) system for the quantitation of circulating serum IgG and IgM antibodies against Hantaan virus.

Reference ELISA assays were set up to measure IgG and IgM antibodies to Hantaan virus. A number of different viral culture extracts and viral constructs were evaluated for use in developing the Optical Bioassay. The viral culture extract was used in developing the IgG assay, and a viral construct was used in developing the IgM assay. The IgG assay was formatted as a 2 step 10 minute test with the viral lysate attached to the OBA test chip and monoclonal anti-human IgG coupled to colloidal gold as the signal enhancer. The IgM assay was formatted as an IgM capture assay with anti-IgM immobilized on the OBA test chip and the Hantaan construct coupled to colloidal gold as the signal enhancer. This required 2 steps and 1 hour. Optimization of the assays should reduce the test to 10 minutes in a 1 step format, but would require additional time to develop more purified viral reagents and optimize assay parameters. A prototype reader was developed during the program, and used to read the assay results. Both test formats were evaluated using 7 positive and 6 negative serum samples. In all cases the OBA results correlated 100% with the ELISA results.

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I. PROJECT OBJECTIVE

The objective of this project was to demonstrate the feasibility of the Optical Bioassay (OBA) system for the determination of serum IgG and/or IgM antibodies to Hantaan Virus.

II. TECHNICAL OBJECTIVES

During the proposed Phase I study we intended to accomplish the following technical objectives:

1. Determine the optimal chemical procedure for activating the silicon surface for protein immobilization.

2. Determine the optimal approach for immobilization of protein onto the activated silicon surface.

3. Develop a prototype instrument for detection of the antigen-antibody binding event using the laser diffraction principle.

4. Demonstrate the feasibility of an OBA Hantaan Virus assay with the optimized chemistry and prototype instrument.

III. REAGENTS AND MATERIALS

Inactivated Hantaan supernatant, negative E6 supernatant, positive serum samples and negative serum samples were provided by the Department of the Army, Medical Institute of Infectious Disease; 4" Silicon wafers were purchased from Polished Corporation of America and subsequently sputtered with aluminum and silicon dioxide by Scientific Coating Labs (Santa Clara, Ca); Aminopropyltriethoxy Silane, sodium periodate, monoclonal anti-human IgG, monoclonal anti-human IgM, and other reagents were obtained from commercial sources without further purification.

IV. EXPERIMENTS AND RESULTS

ACTIVATION OF SILICON SURFACE WITH AMINOPROPYLTRIETHOXY SILANE

Aluminized silicon dioxide surfaces were placed inside a vacuum desiccator and heated to 150° C under vacuum. Aminopropyltriethoxy silane (APTS) was allowed to vaporize into the desiccator through a three way stopcock. The desiccator was kept heated at 150° C for another 16-24 hours. A vacuum pump was connected to the apparatus at the end of the deposition to evacuate the residual APTS. After removal of the residual APTS the silicon wafer was allowed to stand at 150° C for an additional 4 hours and cool to room temperature.

COVALENT ATTACHMENT OF ANTIBODY TO APTS ACTIVATED SURFACE

The antibody was first diluted to 2 mg/ml in 0.2 M acetate buffer, pH 5.0 and cooled in an ice bath to 4° C. A solution of sodium periodate (22.4 mg/ml) in the same buffer was added slowly. After addition the mixture was allowed to stir at 4° C for 1 hour and dialyzed against 0.1 M carbonate buffer. The antibody solution thus oxidized was incubated with APTS activated silicon surface inside a refrigerator overnight. The surface was briefly washed with Tris buffer (0.05 M, Ph 8.5) containing 2.5% sucrose. The residual liquid was removed by spinning the wafer at 2000 rpm to yield the antibody coated silicon wafer.

PHYSICAL ADSORPTION OF PROTEIN TO APTS ACTIVATED SURFACE

APTS activated surfaces are highly hydrophobic. Both antigen and/or antibody can be easily immobilized onto the activated surface through hydrophobic interaction much like the immobilization of protein onto the microtiter wells. To immobilize the protein, an antigen or antibody solution is allowed to incubate on the surface inside a refrigerator overnight. Following the washing and drying procedures as described previously, the protein was easily immobilized onto the silicon surface.

ILLUMINATION OF THE PROTEIN COATED SILICON SURFACE

Protein immobilized silicon wafers were placed under a photomask with alternate parallel opaque and clear lines. The surfaces were illuminated using a Karl Suss Model 40 Mask Aligner with UV light at 254 nm for 100 to 300 seconds. After illumination, the surfaces were used directly or further diced into 4 x 6 mm chips for OBA experiments.

COATING OF MICROTITER WELLS WITH HANTAAN VIRUS ANTIGEN

Both Hantaan cell culture lysates and SF9-Baculovirus preparation were used for the preparation of microtiter wells. After dissolving in the carbonate buffer, the cell lysates solution was first centrifuged at 3000 rpm to remove the insoluble particulates. The clear solution was allowed to incubate in the microtiter wells at room temperature overnight. After rinsing with PBS buffer the wells were further incubated with a carbonate buffer containing 0.5% HSA at room temperature for 45 minutes to block the nonspecific binding sites. The wells were washed again (three times) with the PBS washing buffer and subsequently used in the ELISA assay.

ADEZA ELISA ASSAY FOR SERUM IgG TO HANTAAN VIRUS

Hantaan Virus antigen coated microtiter wells prepared as described were incubated with 150 µl of serum sample at room temperature for 1 hour. The wells were washed three times with PBS washing buffer and incubated with alkaline phosphatase

conjugated anti-human IgG at room temperature for 1 hour. After washing, the wells were finally incubated with phenothalein monophosphate substrate at room temperature for 30 minutes. The OD at 550 nm was determined. It was found that the wells coated with the Hantaan cell lysates performed better than those coated with the SF9 preparation in discriminating between the positive and negative samples (Figure 1 & 2). The cell lysates were subsequently chosen for the remaining studies. In a separate study, we also found that significant improvement was obtained in signal to noise ratio (i.e., positive vs. negative signal) when a fresh batch of cell lysate and a Falcon Pro-Bind microtiter plate were used (Figure 3).

HPLC PURIFICATION OF VIRAL ANTIGEN FROM CELL LYSATES

The cell lysates were first treated with SDS and DTT to solubilize the aggregates followed by dialysis against PBS buffer overnight. The mixture was then purified by fractionation with HPLC (Bio-Rad 800 HRLC System). Four major fractions (Figure 4) were collected and evaluated against the crude cell lysates. It was found that none of the individual fraction performed better than the crude cell lysates.

PREPARATION OF ANTI-HUMAN IgG (or IgM)-COLLOIDAL GOLD CONJUGATE

An aqueous solution of tetrachloroaurate was heated to boiling and reduced with an excess amount of sodium citrate in warm deionized water. The solution turned into a wine-red color within a few minutes to produce the colloidal gold solution. The colloidal gold solution was allowed to cool to room temperature and added rapidly with stirring to a monoclonal anti-human IgG (or IgM) solution at room temperature. After addition, the mixture was stirred at room temperature for another 5 minutes and stabilized with a stabilization buffer containing PEG, PVP, 0.5% HSA and 0.01% thimerosal.

OBA SERUM IgG ASSAY FOR HANTAAN VIRUS

APTS activated silicon wafers were coated with cell lysate and illuminated under UV through the use of mask aligner as described previously. The silicon wafer was diced into 4 x 6 mm chips. The cell lysate coated chips were incubated with serum sample and an excess amount of anti-human IgG-colloidal gold conjugate at room temperature for 10 minutes. After a brief wash with deionized water and drying with a stream of air the diffraction intensities of the chips were determined. It was found that the OBA serum IgG assay can clearly differentiate positive samples from negative samples to Hantaan Virus (Figure 5). A fresh lot of cell lysates was coated and evaluated for OBA assay. It was found that a 5 minutes/5 minutes incubation protocol was sufficient to clearly differentiate the positive and negative serum samples (Figure 6).

PREPARATION OF HANTAAN VIRUS ANTIGEN-COLLOIDAL GOLD CONJUGATE

Antigen 76118 (Lot No. 22) colloidal gold conjugate was prepared follow the same conditions as described for anti-human IgG-colloidal gold conjugate. The antigen-gold conjugate was used successfully in the demonstration of an OBA serum IgM assay for Hantaan Virus.

OBA SERUM IgM ASSAY FOR HANTAAN VIRUS

Attempts to develop an OBA serum IgM assay using the serum IgG protocol as described previously failed due largely to the presence of IgG antibodies in the positive samples. The presence of IgG antibodies hindered the detection of IgM antibodies with the anti-human IgM-colloidal gold approach. An alternative approach with an anti-human IgM antibody coated silicon wafer was investigated. The anti-IgM silicon wafer prepared following the established procedure was illuminated and diced into 4 x 6 mm chips. The chips were allowed to incubate with the serum samples for 30 minutes at room temperature follow by incubation with Hantaan antigen-gold conjugate at room temperature for 30 minutes. The diffraction intensities of the chip were determined. It was found that the assay can clearly differentiate all positive and negative samples (Figure 7).

V. DEVELOPMENT OF A PROTOTYPE OBA READER

In conjunction with other in-house activities, we have developed, designed and fabricated a prototype OBA reader. The major accomplishments in the past few months include: replacement of the bulky and expensive He-Ne laser with a compact and inexpensive solid-state laser, use of optical fiber to efficiently couple the diffraction signal to the detector, use of synchronized detection technique to minimize the effect of laser instability, fabrication of a disposable carrier adaptable to field application interface with a PC for data collection. Current dimensions of the reader are 30 cm x 23 cm x 8 cm which is based on the need for the physician office testing. However with the simplicity of the OBA detection mechanism, it is feasible that a pocket size, battery operated instrument can also be easily manufactured if necessary.

VI. DISCUSSION

The aluminum-silicon dioxide coated silicon wafer has been determined to be the best candidate for the development of the OBA assay. The silicon wafer can be easily activated with silane chemistry through a vapor deposition approach. This approach is quite suitable for a large scale production without the use of any sophisticated or expensive instrumentation. The APTS activated surface containing amino functional groups is highly hydrophobic which can be used for immobilization of either antigen or antibody through physical adsorption or covalent attachment. Denaturation of the immobilized protein can be accomplished with the use of existing integrated circuit

production equipment (i.e., mask aligner photolithographic techniques).

The in-house ELISA assay using PMP as substrate correlates well with the ELISA protocol used by the Army Institute of Infectious Disease. Although purification of the Hantaan Virus antigen through HPLC proved not to be fruitful at this moment, we are confident that a highly purified antigen purification will greatly improve the assay performance.

OBA serum IgG assay for Hantaan Virus has been demonstrated feasible using crude cell lysate coated silicon chips. The sample size used for this feasibility study is limited. We believe a larger sample size study will be appropriate during the Phase II study. A five minute/ 5 minute OBA serum IgG assay for Hantaan Virus was made feasible using a fresh and more antigenic preparation of Hantaan cell lysates. This observation further substantiate our belief of the necessity of a purified and antigenic Hantaan Virus antigen for the final assay development. OBA serum IgM assay for Hantaan Virus has also been demonstrated with a different approach. This approach makes use of an anti-human IgM coated silicon chip to minimize the interference from the serum IgG which presumable will present in a much higher concentration as compared to IgM antibodies. Both OBA serum IgG and IgM Hantaan Virus assays use a two step incubation approach. No attempt to simplify the protocol to a one step assay has been made at this feasibility study stage. However, all other Adeza in-house assays such as a fibronectin assay and milk penicillin assay have all been demonstrated with a one step assay protocol. This one step assay approach involved the use of lyophilized colloidal conjugate in a test tube container. The operator simply adds the sample to the lyophilized colloidal conjugate and incubate the silicon chip with the solution to perform a less than 5 minute OBA assay. In our next phase of study, we will attempt to use this protocol to further our investigation. The incubation time at this moment has been between 10 to 60 minutes for the Hantaan assay. Other Adeza screening assays have been found to perform well even with less than 2 minutes of incubation time.

VII. CONCLUSION

Both the OBA serum IgG and IgM assays have been clearly demonstrated feasible during this Phase I feasibility study. A one step and less than 5 minute assay using the OBA technique is definitely feasible as has been demonstrated with the other Adeza assay development efforts.

The technical objectives of this feasibility study have all been accomplished on time and on budget. We believe the OBA technology can be developed into a useful, simple and inexpensive assay system for the diagnosis of various natural and induced diseases of military importance. We thereby recommend pursuing the Phase II investigation to optimize the reagents, simplify the assay protocol and develop the final products.

VIII. CONFIDENTIALITY

This report and all interactions during the Phase I feasibility study between the Army Medical Institute of Infectious Disease and Adeza Biomedical Corporation should be treated as confidential.

Figure 1

Serum IgG Hantaan Virus ELISA Assay with Cell Lysates

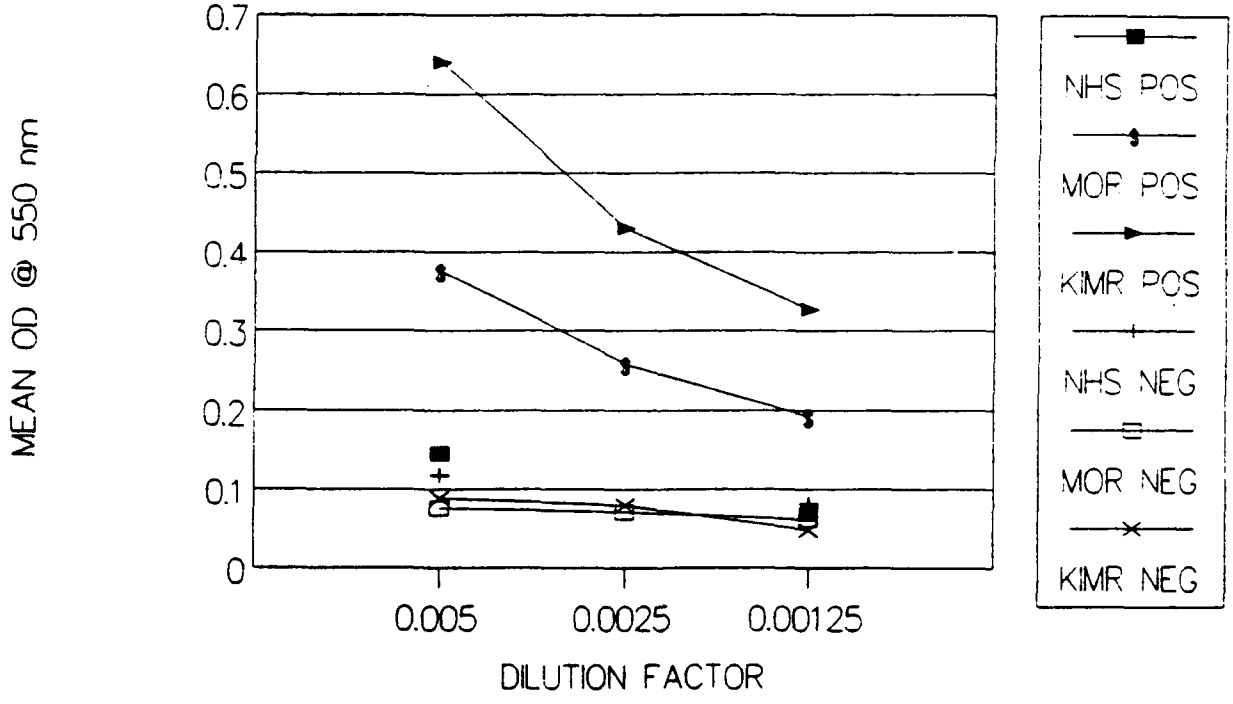

Figure 2

Serum IgG Hantaan Virus ELISA Assay with SF9 Cells

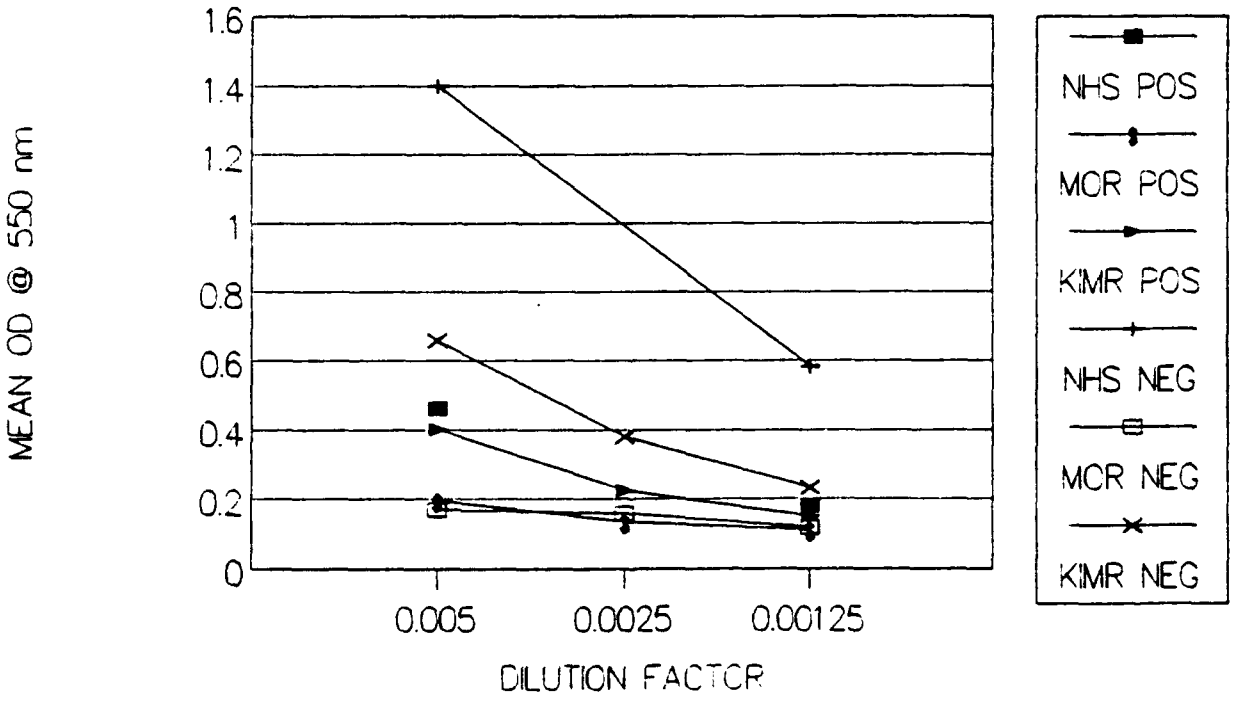

Figure 3

Serum IgG Hantaan Virus ELISA Assay with Pro-Bind Plates

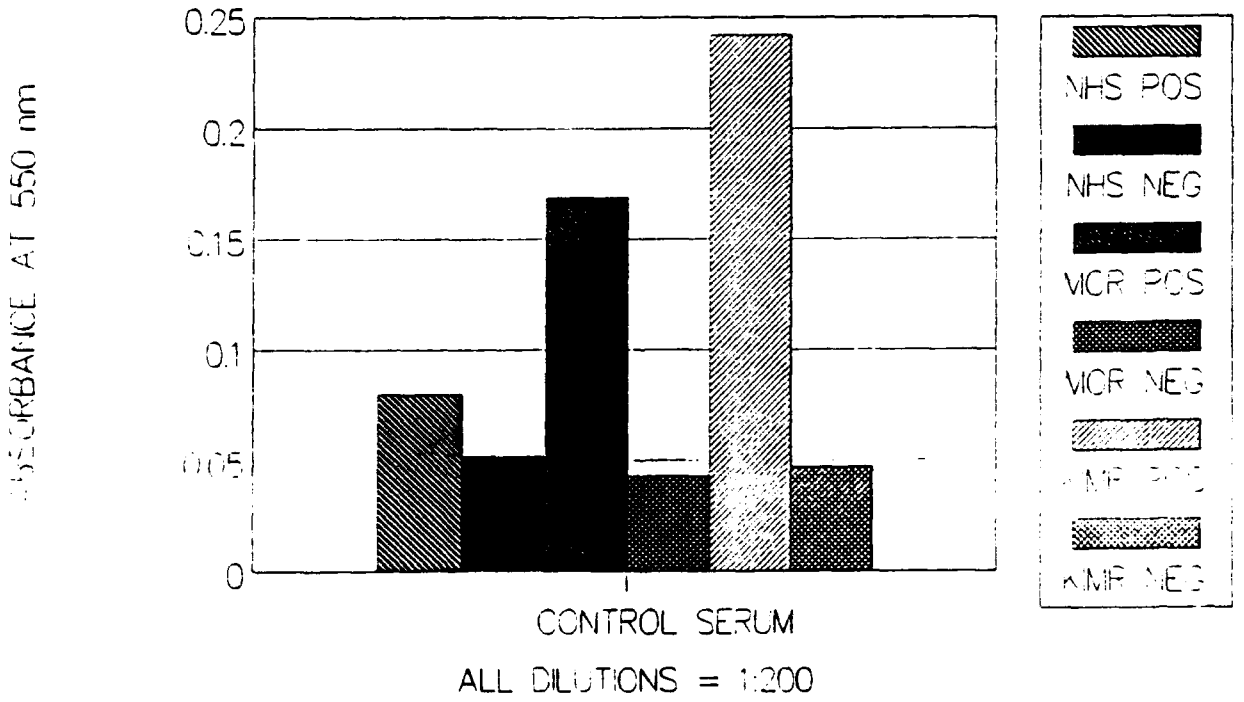

Figure 4

HPLC Purification of Hantaan Virus Cell Lysates

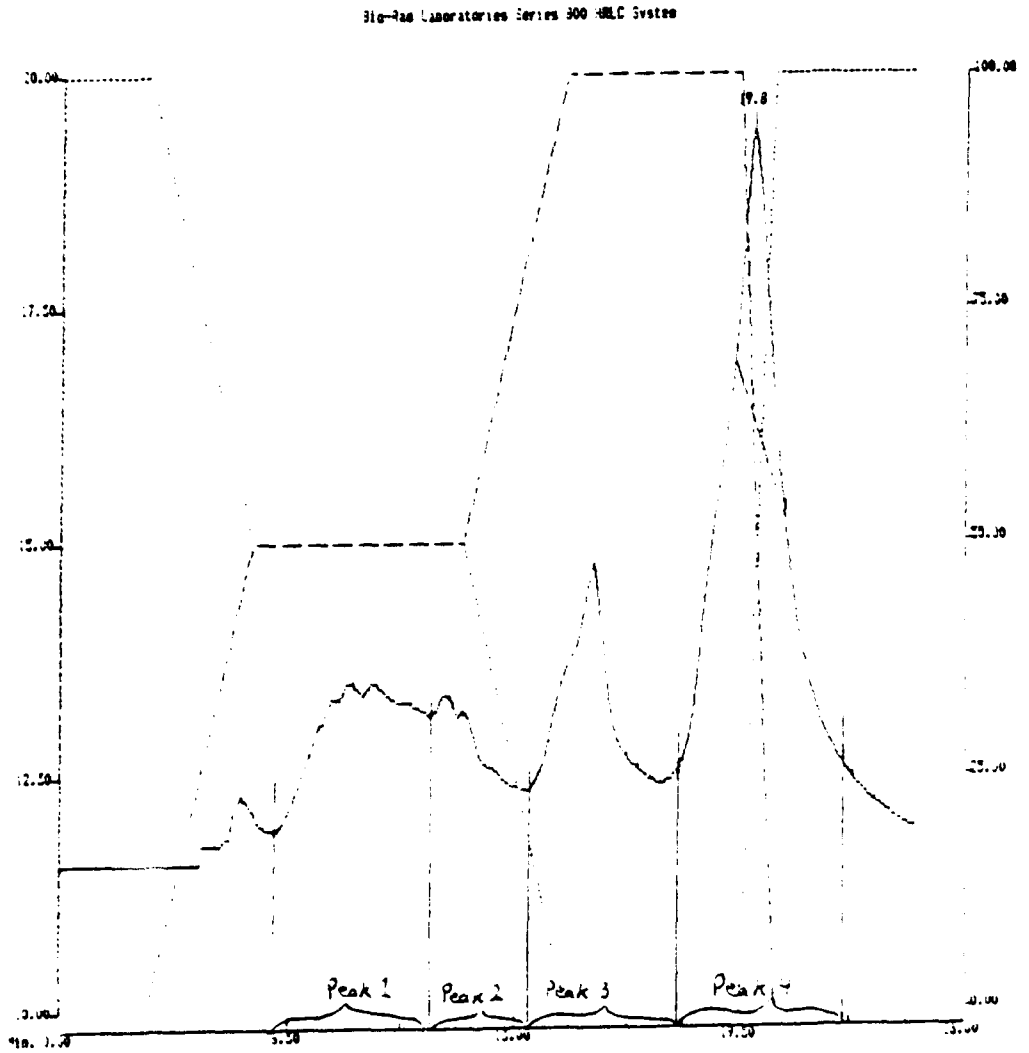

Figure 5

OBA Serum IgG Assay for Hantaan Virus (30/30)

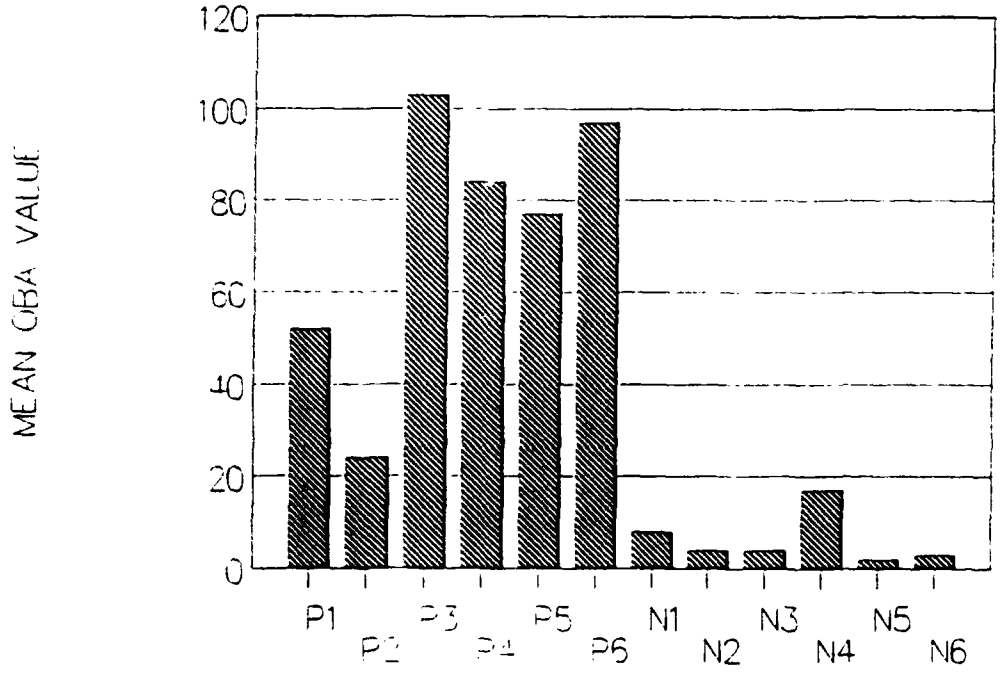

Figure 6

OBA Serum IgG Assay for Hantaan Virus (5/5)

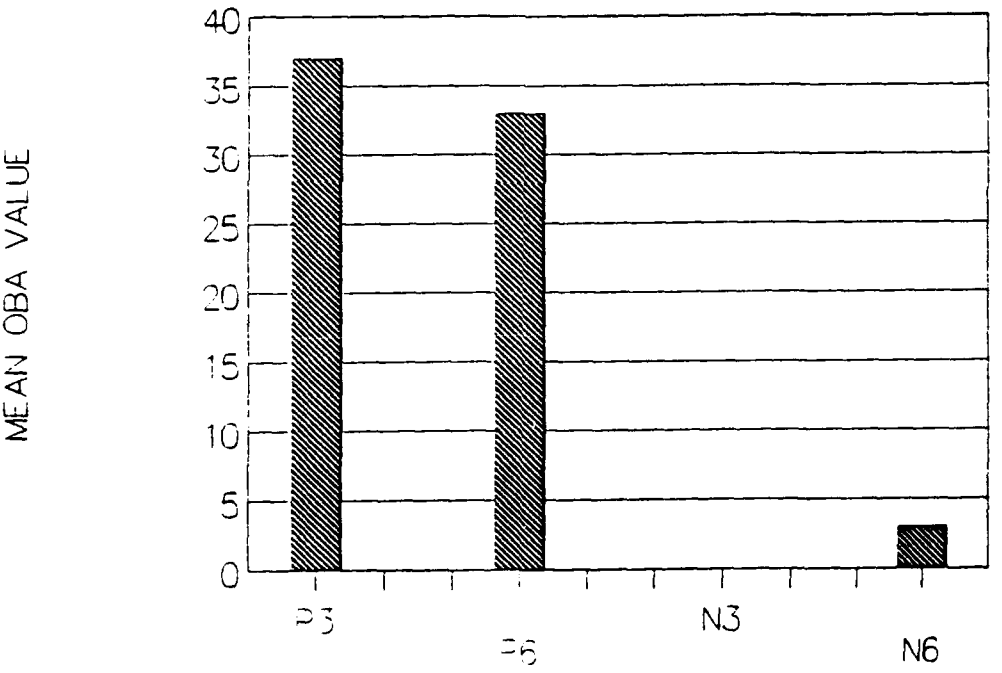

Figure 7

OBA Serum IgM Assay for Hantaan Virus